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The T84 human colonic adenocarcinoma cell line produces mucin in culture and releases it in response to various secretagogues

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The T84 colonic adenocarcinoma cell line, which has been used extensively as a model for studies of epithelial chloride secretion, also produces mucin and secretes it in culture. Electron miscroscopy of fixed sections of cultured cells, along with Immunogold labelling with an antibody to human small intestine (SI) mucin, revealed the presence of goblet-like cells with mucin-containing secretory granules. The mucin was of high molecular mass, had an amino acid composition similar to that of purified human SI and colonic mucins, and competed effectively with SI mucin for binding to the anti-(SI mucin) antibody. A sensitive solid-phase immunoassay specific for intestinal mucins was developed and used to measure mucin secretion by T84 cells. Cultures were treated for 30 min at 37 °C with a number of agents known to cause chloride secretion by T84 cell monolayers and the amount of mucin appearing in the medium was measured. Carbachol (1 mM), A23187 (10 μM), prostaglandin E_1 (PGE_1) (1 μM) and vasoactive intestinal polypeptide (VIP) (0.1 μM) all stimulated mucin release, but histamine (1 mM) had no effect. Whereas VIP is reported to stimulate chloride secretion more strongly than carbachol, it was less effective than carbachol in stimulating mucin secretion. Phorbol 12-myristate 13-acetate (PMA) (0.1–10 μM) also stimulated mucin release strongly, implicating a responsive protein-kinase C-dependent pathway. Additive secretory responses were obtained with combined stimulation by VIP (10 nM–1 μM) and carbachol (1 mM). Responses to stimulation with A23187 (1–10 μM) together with PMA (10 nM–10 μM) suggest that cytosolic Ca^{2+} concentration is a modulator of PMA activity.

INTRODUCTION

The factors which regulate the rate of mucin secretion from intestinal goblet cells are not well defined. In part, this is because it is difficult to examine the functions of an individual cell type in the native mucosa and epithelia, which comprise several kinds of cells (see review by Neutra & Forstner, 1987). Also, the extent to which regulatory mechanisms involved in the secretion of mucin and of electrolytes and water in the digestive tract are interrelated is unclear (see review by Forstner & Forstner, 1986). Recently, colonic cancer lines have been used as models to study the differentiation of intestinal cells *in vitro* and to establish clonal cell lines which express highly differentiated phenotypes. Absorptive and mucus-secreting subclones have been isolated from the human colonic adenocarcinoma cell line HT29 (Augeron & Laboisse, 1984; Huet *et al.*, 1987). Exocytosis from the mucin-secreting subclones (Roumagnac & Laboisse, 1987; Phillips *et al.*, 1988) has been shown to be responsive to cholinergic stimulation. Another human colonic adenocarcinoma cell line, T84, has been used extensively as a model system for the study of epithelial electrolyte transport and its regulation by various hormones and neurotransmitters (Dharmsathaphorn *et al.*, 1984; Madara *et al.*, 1987). When grown as a monolayer with tight junctions, this cell line secretes chloride vectorially through apical channels in response to a variety of secretagogues, such as vasoactive intestinal polypeptide (VIP), carbachol and the calcium ionophore A23187. Brady *et al.* (1984) showed that T84 cells cultured as a mixture of aggregates and monolayers contained granules which reacted, as shown by immunofluorescence, with an antibody raised in rabbits in our laboratory against human small intestinal (SI) mucin. These observations suggest that cultures of T84 cells may contain two populations of differentiated epithelial cells which are either electrolyte- and water-secreting or mucin-producing, and thus could be used to compare regulatory pathways involved in the secretion of electrolytes and of mucin.

In this report, we provide evidence that cultures of T84 cells contain goblet-like cells with large mucin-containing granules, and that the mucin is of large molecular mass and has a density and amino acid composition similar to those of previously characterized human intestinal mucins. We have also devised a simple solid-phase immunoassay which is specific for mucin and suitable for measuring mucin production by cells in culture. Using our T84 cultures, which contain chloride-secreting (and therefore water-secreting) cells along with mucin-secreting cells, we have studied the effects on mucin release of a variety of agents known to stimulate chloride secretion by T84 monolayers and/or mucin secretion from other cell types.

MATERIALS AND METHODS

Cell lines and cell culture

T84, LS174T and HCMC cell lines were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. T84 cells were grown on plastic in a 1:1 mixture of Dulbecco's modified Eagle's medium with 4.5 g of D-glucose/litre and Ham's F12 Nutrient mixture (Gibco, Grand Island, NY, U.S.A.) containing 5% fetal bovine serum (Bocknek Labs Inc., Toronto, Canada). LS174T cells were grown in Eagle's minimum essential medium with non-essential amino acids in Earle's balanced salts solution containing 10% fetal bovine serum. HCMC cells were grown in the same media as LS174T cells but with the fetal bovine serum concentration increased to 25%. All culture media contained penicillin (100 units/ml) and streptomycin (100 μg/ml) (Gibco). Cultures were maintained at 37 °C in a humidified atmosphere of air/CO_2 (19:1, v/v) and were passaged by treatment of the adherent cells at 37 °C for 7–10 min with 0.05%

Abbreviations used: SI mucin, small intestinal mucin; PGE_1, prostaglandin E_1; VIP, vasoactive intestinal peptide; PMA, phorbol 12-myristate 13-acetate; CF, cystic fibrosis; PAS, periodic acid–Schiff; PBS, Dulbecco's phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride.

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trypsin/0.53 mM-EDTA (Gibco), pH 7.4. The reaction was terminated by addition of 1.0 ml of fetal bovine serum. Cells were immediately pelleted by centrifugation (250 *g* for 3 min) resuspended in culture medium and seeded at 4×10^{-4} cells/cm^2.

Human mucins and antibodies

Mucins from a normal small intestine (SI) and the colon of a cystic fibrosis (CF) patient were purified according to Mantle *et al.* (1984*a*). Antibodies to normal human SI mucins and CF colonic mucin were 40% (w/v) $(NH_4)_2SO_4$ fractions of immune rabbit sera. The specificity of the anti-(SI mucin) antibody has been presented in earlier reports (Qureshi *et al.*, 1979; Mantle *et al.*, 1984*b*).

Histology

At 70% confluence, cells were scraped off a culture dish using a rubber policeman without removing the medium and pelleted by centrifugation at 1500 *g* for 5 min. Medium was removed and the pellet fixed in 2.0 ml of 10% formalin. Cells were embedded in paraffin, sectioned at 6 μm and stained with Alcian Blue (pH 2.5)/PAS (periodic acid–Schiff) to detect the presence of acid and neutral glycoconjugates (Pearse, 1968). To monitor glycogen, tissue sections were treated for 20 min at room temperature with 0.5% amylase (Sigma Chemical Co., St. Louis, MO, U.S.A.) and washed thoroughly with distilled water prior to staining. Hepatic sections, containing hepatocytes with intracellular glycogen, served as positive controls.

For routine monitoring for the presence of mucus, cells were fixed in the tissue culture dish by addition of 1.0 ml of Rossman's fixative (saturated picric acid in 100% ethanol/formaldehyde, 9:1, v/v) rinsed with tap water and then stained with PAS (Pearse, 1968).

Electron microscopy and Immunogold localization of mucin

Cells seeded at $(2–4) \times 10^5$/well were grown for 5–7 days in 6-well (35 mm) culture dishes (Linbro; Flow Labs Inc. McLean, VA, U.S.A.) containing 2 ml of growth medium/well, with a change of medium on the 3rd day of culture. The spent medium was removed and the cells were washed twice with Dulbecco's phosphate-buffered saline (PBS), pH 7.4, and then fixed for 1 h at room temperature with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M-sodium phosphate buffer, pH 7.4. Cells were then gently scraped off dishes using a rubber policeman. The cells from two wells were placed in three 1.5 ml Eppendorf tubes which contained 1 ml of fixative, and centrifuged for 4 min at 11500 *g* to form pellets. The latter were stored at 4 °C for about 2 h and post-fixed with 1% osmium tetroxide. The pellets were dehydrated through alcohols, then rinsed with acetone prior to embedding in Epon/Araldite which was polymerized overnight at 60 °C. Sections (about 90 nm thick) were mounted on nickel grids and treated with 10% H_2O_2 at room temperature for 5 min. Following a rinse with megapure water, the sections were floated face-down on drops of 3% BSA (essentially globulin-free; Sigma) at room temperature for 30 min. The sections were then floated out on drops of anti-mucin antibody at a dilution of 1:400 (v/v) in PBS (pH 7.2) containing 1.0% BSA in a humidity chamber overnight at 4 °C, followed by 1 h at room temperature on the following day. Negative controls, using no antibody, an immunoglobulin preparation from preimmune rabbit serum or an irrelevant antibody (anti-insulin), were also carried out. Following washing with PBS, the sections were floated face-down on drops of Protein A–gold complex (18 nm gold particles) for 1 h at room temperature. The sections were then jet-rinsed with megapure water, air-dried and counter-stained using uranyl acetate and Sato's lead. Sections were viewed on a Philips 400 transmission electron microscope.

Solid-phase immunoassay for mucin

Mucin-containing samples in a volume of 200 μl or less were slotted on to nitrocellulose in a Slot Blot Minifold II Apparatus (Schleicher and Schuell, Keene, NH, U.S.A). The nitrocellulose sheet was removed, and the remaining binding sites were blocked for 1 h at room temperature with 3% BSA (essentially fatty-acid free; cat. no. A7030, Sigma) in 10 mM-Tris/150 mM-NaCl buffer, pH 7.4 (TBS). The sheet was incubated overnight at 4 °C in anti-mucin antibody in TBS containing 3% BSA, followed by washing with TBS at room temperature to remove excess antibody. It was then incubated for 1.5 h at room temperature with protein A–horseradish peroxidase conjugate (Bio-Rad, Richmond, CA, U.S.A.) diluted 1:3000 (v/v) in TBS containing 3% BSA. Excess conjugate was removed by washing in TBS. Horseradish peroxidase colour development reagent containing 4-chloro-1-naphthol (Bio-Rad) (30 mg) was dissolved in 10 ml of methanol in the dark. Ice-cold 30% H_2O_2 (30 μl) was added to 50 ml of TBS at room temperature and then added to the methanol solution above and mixed. The nitrocellulose sheet was immediately incubated in this solution in the dark for about 10 min at room temperature to allow colour development, then rinsed in distilled water and blotted on filter paper to dry. The sheet was scanned using a densitometer (Ultrascan XL Laser, Pharmacia LKB, Uppsala, Sweden) to measure the colour intensity of each slot. Standard curves were constructed using a range of concentrations of mucin and anti-mucin and were used to determine the amount of mucin in test samples. For assays involving T84 mucin, SI mucin (5–100 ng as protein) was used as standard with its antibody at a dilution of 1:12000 (v/v).

Competitive inhibitions of binding of SI mucin and T84 culture extracts to the anti-(SI mucin) antibody

Aliquots (1 ml) of the anti-(SI mucin) antibody at a final dilution of 1:24000 (v/v) were incubated with increasing amounts of either SI mucin (100–500 ng of protein) or a supernatant. The supernatant was obtained by centrifugation of a T84 cell sonicate in TBS at 100 *g* for 5 min at 4 °C and contained 125–625 ng of mucin protein (as determined by immunoassay). Incubations were carried out in 16 mm well tissue culture plates (Costar, Cambridge, MA, U.S.A.) for 1 h at 37 °C followed by 4 h at room temperature. Control incubations contained antibody but no antigen additions. Meanwhile, samples of mucin (40 ng of protein) or T84 supernatant containing 50 ng of mucin protein (determined by immunoassay) were slotted on to nitrocellulose and blocked with TBS containing 3% BSA for 1 h at room temperature. The individual slots were then cut out and placed in the well containing the various antibody–antigen mixtures and incubated overnight at 4 °C. After washing the slots to remove unbound antibody, the amounts of antibody bound were estimated using substrate colour development and densitometry as in the regular immunoassay. Values obtained for samples incubated with antibody which had been exposed to mucin or T84 supernatant were compared with those obtained using the control antibody solutions, and the percentage inhibition due to preincubation of the antibody with antigens was determined.

Preparations of T84 cell mucin

Mucin was isolated by a modification of previously described methods (Wesley *et al.*, 1985). Approx. 3×10^8 cells were grown to 70% confluence in 15 cm culture dishes, harvested using a rubber policeman and homogenized in 15 vol. of ice-cold medium containing 0.2 M-NaCl, 0.02% NaN_3, 1 mM-phenylmethanesulphonyl fluoride (PMSF), 1 mM-benzamidine and 5 mM-Na_2EDTA (pH 7.2). The following procedures were performed at 4 °C. The homogenate was centrifuged at 1500 *g* for 10 min to

remove cell debris, then at 105000 *g* for 1 h, and the resulting supernatant was dialysed for 24 h against distilled water and then lyophilized. This material was chromatographed on Sepharose Cl-4B with 0.2 M-NaCl as an eluent. The void volume fractions, which contained the mucin, were pooled and subjected to density-gradient ultracentrifugation in CsCl (starting density 1.42 g/ml) for 48 h at 105000 *g*. Fractions of 2 ml were collected from each 40 ml gradient, and 50 μl aliquots from each fraction were assayed for mucin by immunoassay (see above). Mucin-containing samples were dialysed against distilled water and lyophilized.

Enzymic degradation

Samples, each containing 10 μg of T84 mucin protein, were subjected to the following enzyme digestions: Pronase (Protease type XIV, Sigma) (5 μg/ml) at 37 °C for 24 h in Dulbecco's PBS (pH 7.4) minus Ca^{2+} and Mg^{2+}; chondroitinase ABC, 0.4 units/ml in 0.1 M-Tris/acetate (pH 8.0) at 37 °C for 5 h; hyaluronidase (*Streptomyces*), 10 units/ml in 0.02 M-sodium acetate (pH 6.0) containing 150 mM-NaCl for 16 h at 37 °C; heparitinase, 5 units/ml in 0.1 M-sodium acetate (pH 7.0) containing 1 mM-$CaCl_2$ for 5 h at 42 °C. Chondroitinase ABC, hyaluronidase and heparitinase were from Miles Scientific Co., Naperville, IL, U.S.A. A control sample of mucin was incubated under the same conditions used for hyaluronidase digestion but without the addition of enzyme. At the end of the incubation period, samples were chilled in ice and PMSF was added to give a final concentration of 1 mM. The samples were then chromatographed on Sepharose Cl-4B at 4 °C and eluted with 10 mM-Tris/HCl, pH 7.4, containing 0.2 M-NaCl, and 1 ml fractions were collected. Aliquots (100 μl) were taken for immunoassay to measure the amount of mucin in each fraction.

Chemical degradation

Reductive cleavage of *O*-glycosidic linkages by β-elimination of a T84 mucin was carried out in 50 mM-NaOH containing 1.0 M-$NaBH_4$ for 24 h at 45 °C. A control, containing T84 mucin in distilled water, was incubated under the same conditions. After incubation, the samples were chilled in ice and the $NaBH_4$-treated samples were neutralized with acetic acid. The mucin content of control and treated samples was then estimated by immunoassay.

Amino acid and hexosamine analysis

Amino acid analysis was carried out using a Waters Pico-tag amino acid analysis system with gas-phase hydrolysis in 6 M-HCl and 0.05% Phenol at 110 °C for 24 h. For hexosamine analysis, a 6 h hydrolysis was used.

Mucin secretion assays

T84 cells from passages 3 to 13 were used in these experiments. Cells were seeded into 6-well (35 mm) culture dishes (Linbro; Flow Labs), each well containing about 2×10^5 cells in 2 ml of culture medium. The cells were given fresh medium on the third and fifth days of culture, and 5–7 day-old cultures (70–95% confluent) were used in secretion assays as follows. Spent medium was removed and the cells were washed twice with 1 ml of warm (37 °C) Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (1:1) containing 15 mM-Hepes (pH 7.4) and $NaHCO_3$ (1.2 g/l) (Gibco). A further 1 ml was then added to each well and the cells were allowed to equilibrate for 15 min at 37 °C in a CO_2 incubator. The medium was removed and 1 ml of fresh medium, with or without potential secretagogues, was added to each well and the incubation was continued, unless otherwise specified, for 30 min at 37 °C. The medium was harvested into 1.5 ml Eppendorf tubes containing 10 μl of 100 mM-PMSF and mixed, and aliquots were removed for immunoassay of mucin content. Portions (1 ml) of ice-cold 10% trichloroacetic acid were then added to each well, and the cell protein was allowed to precipitate overnight at 4 °C. The precipitates were washed with 10% trichloroacetic acid and dissolved in 1 ml of 0.1 M-NaOH. Aliquots (25 μl) were then used for protein analyses (Lowry *et al.*, 1951) using BSA (Fraction V, Sigma) as standard. The amounts of mucin protein secreted were expressed as pg/μg of cellular protein.

Chloride efflux assays

T84 cells were cultured as described for mucin secretion assays and measurements of chloride efflux were carried out using the method of Mandel *et al.* (1986) for T84 monolayers. Briefly, cell cultures were loaded with $^{36}Cl^-$ by a 3 h incubation at room temperature in KCl buffer, washed twice with 2 ml of sucrose buffer and incubated for 1 h in 1 ml of sucrose/ouabain buffer containing 0.1 mM-furosemide. Efflux was then initiated by aspiration of the sucrose/ouabain buffer and addition of 1 ml of a buffer containing 120 mM-sodium gluconate, 10 mM-potassium gluconate, 10 mM-NaCl, 20 mM-Hepes/Tris, pH 7.5, 1 mM-$MgSO_4$, 0.5 mM-ouabain and 0.1 mM-furosemide in the presence or absence of potential secretagogues. At various times, efflux was terminated and the loss of intracellular isotope was measured. The amounts of remaining intracellular chloride were expressed as nmol/mg of cellular protein.

Chemicals

PMSF, VIP, carbachol (carbamoylcholine), PMA, histamine dihydrochloride, prostaglandin E1 (PGE_1), the calcium ionophore A23187 (free acid) and cholera toxin were from Sigma. Atropine was from Aldrich, Milwaukee, WI, U.S.A.

RESULTS

Histology and electron microscopy

Phase-contrast microscopy revealed that the T84 cells grew as a mixture of spheroid aggregates and monolayers on top of a basement membrane. Alcian Blue/PAS staining of fixed cultures showed that monolayer cells contained a small number of PAS-positive granules. Some of these granules were found to contain glycogen, since diastase treatment decreased the number of granules which stained positively. As the cell aggregates grew larger, more PAS-positive, diastase-resistant, material appeared intracellularly and on the surface of the cells, and sometimes a glandular arrangement of cells secreting PAS-positive material into an intracellular lumen could be seen. The PAS-positive surface coat was easily removed by washing twice in PBS buffer, using gentle agitation on a rotary shaker for 1 min with each wash. Alcian Blue staining of the monolayers was negative and of the aggregates very weak. Electron micrographs of fixed sections showed the presence of some cells with the characteristics of mucin-producing goblet cells of normal colon. Fig. 1(*a*) shows one such cell which contains large secretory granules and is located next to a cell devoid of these granules. Immunogold localization of the anti-(SI mucin) antibody, to demonstrate the presence of mucin, was predominantly in the large granules and was absent from the cytoplasm of the adjacent granule-free cell (Fig. 1*b*). From a micrograph which was photographed at higher magnification (Fig. 1*c*), it can be seen that the gold particles were evenly distributed throughout the granules, and granular membranes were not labelled significantly. Fusion of the membranes of several granules with the apical plasma membrane can be seen and there is evidence of labelled material being released at the apical surface (Figs 1*b* and 1*c*). Labelling was not observed in controls where the anti-mucin antibody was replaced

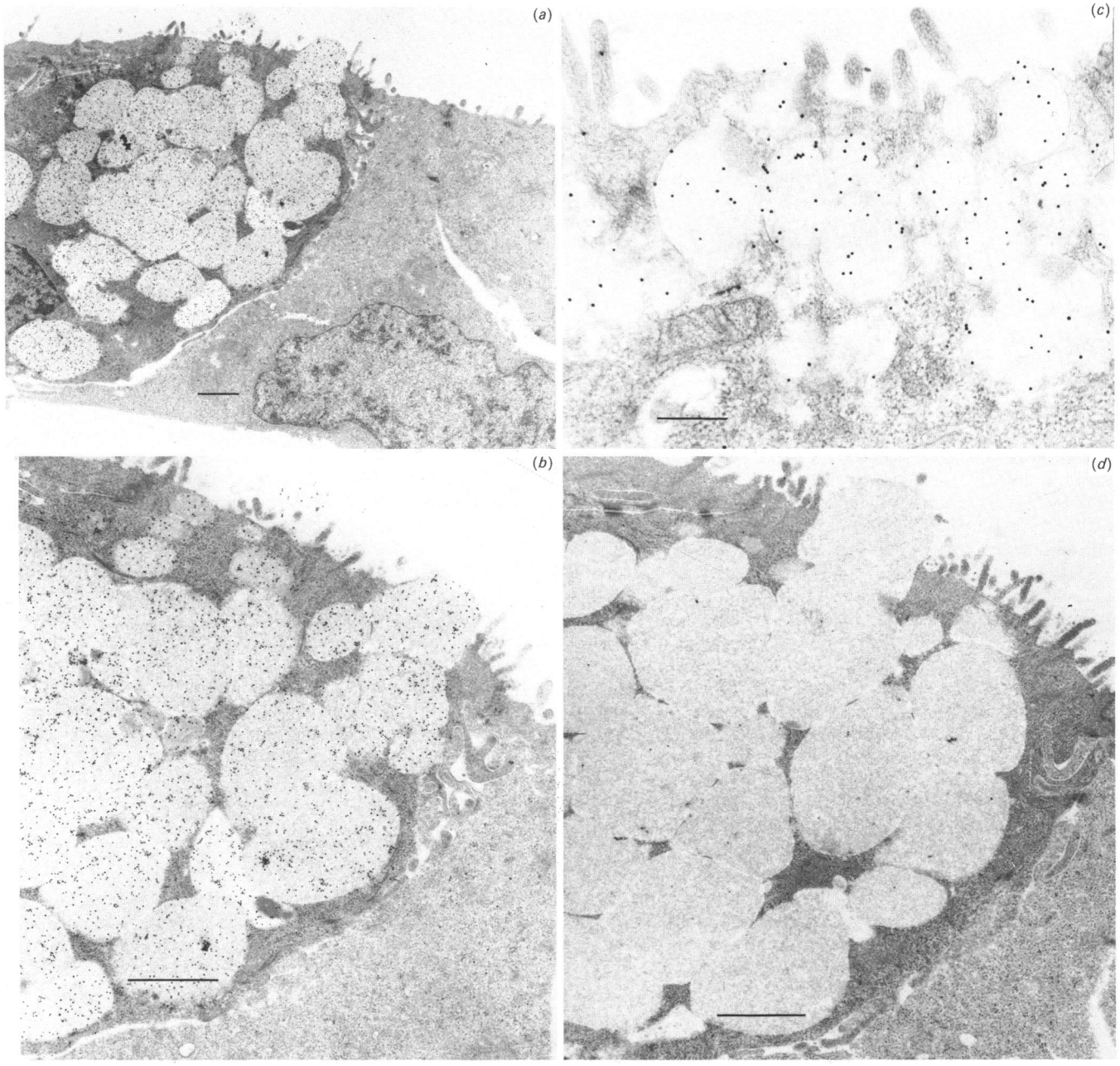

Fig. 1. Electron micrographs of goblet-like cells

(*a*) Section of cells showing a goblet-like cell and Immunogold localization of the antibody to SI mucin at a 1:400 (v/v) dilution; bar = 1 μm. (*b*) Same cell; bar = 1 μm. (*c*) Apical area from another cell; bar = 0.6 μm. (*d*) Anti-mucin antibody was replaced by an IgG fraction from preimmune serum at a 1:400 (v/v) dilution; bar = 1 μm.

by an immunoglobulin fraction from preimmune serum (Fig. 1*d*) or by an irrelevant antibody (anti-insulin). From examination of the sections, we noted that about 5% of the cells contained mucin granules and about 30% of these were filled with granules like typical goblet cells. Using different batches of serum for culture did not change markedly the percentage of cells containing mucin granules. In addition, when the cell line was cultured in serum-free medium reported to stimulate growth of T84 cells as gland-like structures (Murakami & Masui, 1980), no significant increase in the number of mucus-secreting cells was noted.

Immunoassay for mucin

Using SI mucin and its polyclonal antibody, a sensitive solid-phase immunoassay for mucin was established (see the Materials and methods section). Fig. 2(*a*) shows a composite standard curve from five experiments over a range of 10–100 ng of SI mucin (protein) using an antibody dilution of 1:12000 (v/v). A control in which the anti(-SI mucin) antibody was replaced with an immunoglobulin fraction from preimmune serum at the same protein concentration gave negative results (Fig. 2*a*). A similar standard curve using colonic mucin and the antibody to it was constructed. Cross-over experiments revealed that the colonic and SI antigens were recognized well by both antibodies, but curves obtained with one antibody using the two different mucin antigens over the same concentration range were not exactly the same, indicating that the two mucin preparations did not have completely identical epitopes.

Supernatants prepared from T84 cell sonicates and

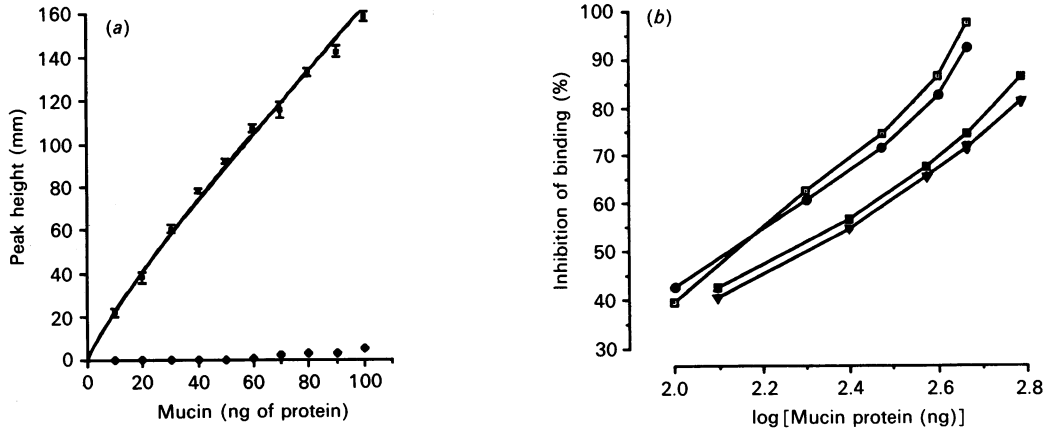

Fig. 2. Mucin immunoassay: standard curve (*a*) and competitive inhibition (*b*)

(*a*) Standard curve showing means ± S.E.M. of peak heights obtained from densitometer scans using increasing amounts of human SI mucin with the antibody to the mucin at a dilution of 1:12000 (v/v) (■) or with an IgG fraction prepared from preimmune serum (◆). (*b*) Inhibition of binding to mucin (40 ng of protein) or T84 supernatant (50 ng of mucin protein) by preincubating the antibody at a 1:24000 (v/v) dilution with increasing amounts of mucin (100–500 ng of protein) or T84 supernatant (125–625 ng of mucin protein). Shown are: mucin (□) or T84 supernatant (●) with antibody preincubated with mucin; and mucin (■) or T84 extract (▼) with antibody preincubated with T84 supernatant.

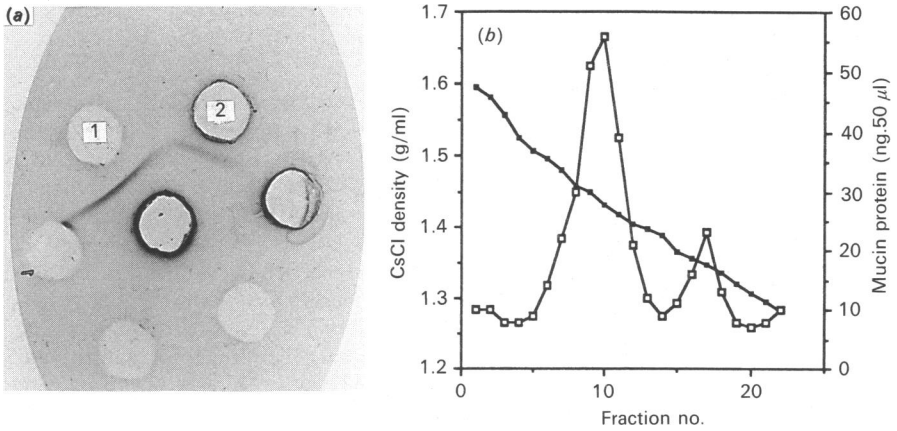

Fig. 3. Immuno-cross-reactivity (*a*) and buoyant density (*b*) of mucin formed by T84 cells

(*a*) Ouchterlony diffusion gel. The SI mucin was in well 1, the T84 supernatant in well 2 and the antibody to SI mucin in the centre well. (*b*) CsCl density-gradient fractionation. Fractions of 2 ml were collected from a 40 ml gradient, and 50 μl aliquots of each were assayed for immuno-reactive mucin protein (ng/50 μl) (□). ■, CsCl density (g/ml).

homogenates and spent culture medium reacted with both SI and colonic antibodies when tested by immunoassay. Similar results were obtained using homogenate supernatants from the mucin-secreting cell line LS174T. Since the antibody to SI mucin has been well-characterized (Qureshi *et al.*, 1979; Mantle *et al.*, 1984*b*) and reacted strongly with T84 supernatants, we decided to use this antibody and its antigen as standards for routine assays and subsequent experiments.

To check that the immunoreactive component in the T84 cell supernatants was able to compete effectively with the SI mucin antigen for binding to the SI mucin antibody, inhibition experiments were performed. Fig 2(*b*) shows that blocking the antibody with increasing amounts of SI mucin progressively inhibited its binding to either fresh SI mucin or T84 supernatant to a similar extent. Likewise, preincubation with T84 supernatant competitively inhibited antibody binding to both SI mucin and T84 supernatant. Thus the cell supernatant component reacted with antibody via antigenic determinants which were similar to those of the SI mucin. This was demonstrated further by Ouchterlony gel diffusion in which a line of identity between SI mucin and the T84 supernatant was obtained using the anti-(SI mucin) antibody (Fig. 3*a*).

A linear plot with a slope like that of the mucin standard was obtained when the amounts of mucin present in samples (50–200 μl) of medium from secretion assays were measured by immunoassay, and densitometer peak height was plotted against sample volume. SI mucin samples (30 ng) were estimated accurately when added to medium samples known to contain various amounts of T84 mucin protein (5–30 ng). Thus the immunoassay is suitable for quantifying mucin secreted by cells in culture. For measurement of the mucin content of cell homogenates or sonicates, samples had to be diluted in TBS until the protein content was about 10 μg/ml (measured by the method of Lowry *et al.*, 1951), and less than 100 μl of this was slotted to avoid interference by non-mucin constituents.

Characterization of mucin produced by cultured T84 cells

To show that the immunoreactive material present in the T84 cell cultures had the characteristics of a mucin, antigenic material was purified from homogenate supernatants by Sepharose Cl-4B

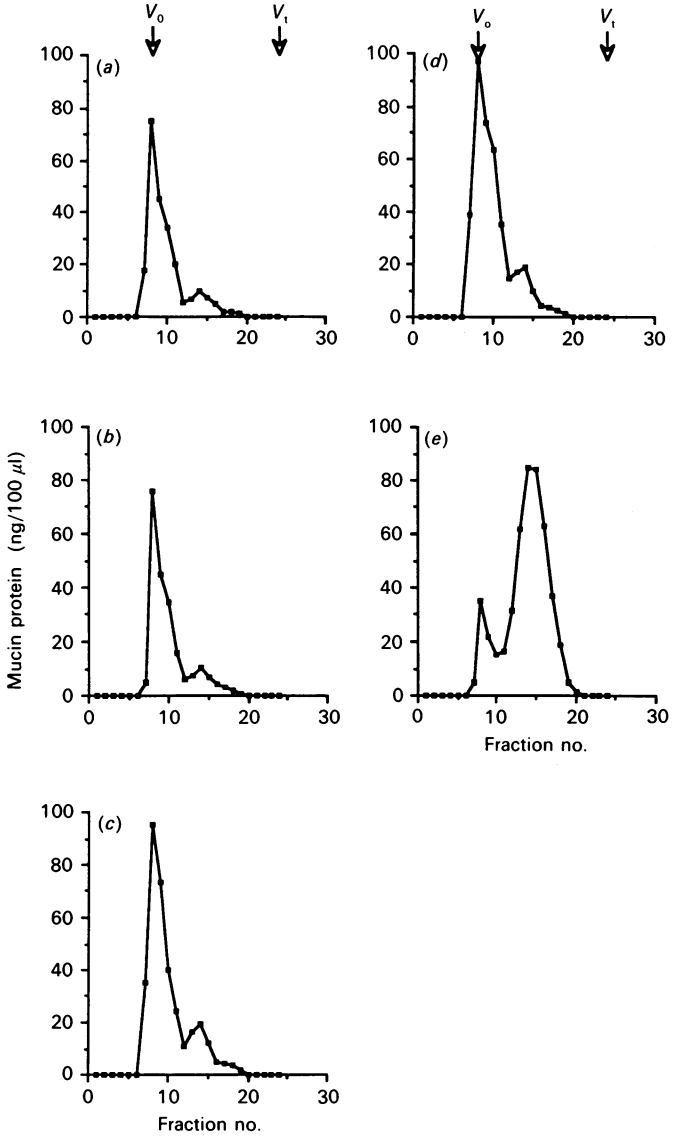

Fig. 4. Sepharose 4B chromatography of control and enzymically treated T84 mucin

Samples of a T84 mucin preparation containing 10 μg of mucin protein were untreated (*a*) or treated with hyaluronidase (*b*), chondroitinase ABC (*c*), heparitinase (*d*) or Pronase (*e*), and then rechromatographed on Sepharose Cl-4B and the mucin content of each fraction measured.

chromatography followed by CsCl density-gradient ultracentrifugation. The material was of high molecular mass, since it eluted predominantly in the void volume on Sepharose 4B chromatography (Fig. 4*a*). On SDS/PAGE, using a 4.0% stacking gel and a 7.5% separating gel (Laemmli, 1970) followed by Western blotting with the antibody (1:600 dilution, v/v), the immunoreactive material was present in the stacking gel but did not enter the separating gel (results not shown). CsCl density-gradient ultracentrifugation followed by fractionation gave two immunoreactive peaks (Fig. 3*b*). The major peak had a buoyant density of 1.42–1.45 g/ml, whereas the minor fraction had a peak density of 1.35 g/ml. For both peaks, the amino acid composition suggested some contamination by non-mucin protein. Sufficient material from the high-density peak was collected, however, for further chromatography on Sepharose Cl-2B using 0.2 M-NaCl at 4 °C as an eluant. Amino acid analysis of the V_0 fraction gave a composition typical of mucin (Table 1), rich in threonine and

Table 1. Amino acid composition of mucins

Source of mucins: (*a*) colon (CF); (*b*) T84; (*c*) small intestine (Wesley *et al.*, 1985); (*d*) LS174T xenograft (Byrd *et al.*, 1988).

Component	Composition (mol %)			
	(*a*)	(*b*)	(*c*)	(*d*)
Asp	5.19	6.88	6.0	4.3
Glu	7.12	8.07	7.3	6.4
Ser	7.72	9.47	9.4	12.9
Gly	7.77	7.91	7.1	6.2
His	1.08	1.98	2.1	3.2
Arg	1.64	2.46	1.9	2.0
Thr	30.20	25.20	24.3	28.4
Ala	3.89	5.10	5.6	5.6
Pro	17.05	12.92	12.0	14.1
Tyr	0.88	1.65	1.9	1.2
Val	4.22	4.85	4.9	4.6
Met	1.28	1.71	0.6	–
Cys*	1.46	–	Trace	–
Ile	3.92	3.06	4.4	3.2
Leu	4.33	3.95	6.1	4.5
Phe	0.19	1.48	3.9	1.6
Lys	2.05	3.31	2.5	3.5

* Not accurate

similar to colonic and SI mucins prepared in our laboratory and to LS174T mucin isolated from mouse xenografts (Byrd *et al.*, 1988). Alcian Blue staining at acid pH may be stronger in LS174T cells (Hand *et al.*, 1985) than our results suggest for T84 cells, raising the possibility that the degree of post-translational glycosylation and sulphation may be different. Hexosamine analysis carried out at the same time as amino acid analysis gave an *N*-acetylgalactosamine/*N*-acetylglucosamine ratio of 1.54, and the *N*-acetylgalactosamine/(serine + threonine) ratio was estimated to be about 0.74. Unfortunately, there was not sufficient material for g.l.c. analysis to determine carbohydrate composition.

Experiments to demonstrate that the antibody used for immunoassay did not recognize proteoglycans or glycosaminoglycans were carried out using methods similar to those described by Kim *et al.* (1985) for hamster tracheal cell cultures. Tumour cell mucin was prepared and samples were treated enzymically. Hyaluronidase, chondroitinase ABC and heparitinase did not cause changes in the elution profile of immunoreactive T84 mucin on Sepharose Cl-4B (Figs. 4*a*–4*d*). In addition, chondroitin sulphate (Sigma) and heparin sulphate (Miles) (10 μg of each) were negative by immunoassay. Homogenate supernatants were also prepared from the PAS-negative (i.e. non-mucin-producing) human colonic HCMC cell line and were negative for mucin by immunoassay. Taken together, these results indicate that the assay did not detect non-mucin material such as proteoglycans.

As reported previously for SI mucin (Mantle *et al.*, 1984*b*), digestion of the T84 mucin by Pronase resulted in a major shift of immunoreactive material into the included volume of the 4B column (Fig. 4*e*). Continuing the Pronase digestion beyond 24 h resulted in a progressive loss of antigenicity. Alkaline borohydride treatment which cleaves *O*-glycosidic linkages and disulphide bonds known to be present in mucin molecules (Aminoff *et al.*, 1980) resulted in a loss of about 85% of immunoreactivity (results not shown).

Stimulation of mucin secretion

The effect on mucin secretion by T84 cells of agents known to stimulate (1) chloride and water secretion by T84 monolayers or

Table 2. Stimulation of mucin release from T84 cell cultures

Cells were washed twice with medium warmed to 37 °C, then allowed to equilibrate for 15 min at 37 °C in fresh medium. This medium was replaced by medium with or without secretagogue and the cells were incubated at 37 °C for 30 min. The total amounts of mucin released into the medium during the 30 min period were measured by immunoassay as described in the Materials and methods section. Mucin release is given as means ± S.E.M. ($n = 6$). For stimulation results, control = 100% in each experiment.

Expt.	Secretagogue	Concentration (M)	Mucin protein (pg/μg of cell protein)	Stimulation (%)
1.	None	–	103.8 ± 2.1	100
	Carbachol	10^{-3}	324.2 ± 7.3	312
	A23187	10^{-5}	431.9 ± 15.3	416
	VIP	10^{-7}	216.5 ± 7.4	209
2.	None	–	141.2 ± 4.3	100
	A23187	10^{-6}	245.7 ± 11.9	174
	A23187	10^{-5}	623.2 ± 19.5	441
3.	None	—	154.2 ± 6.7	100
	Carbachol	10^{-4}	288.8 ± 5.3	187
	Carbachol	10^{-3}	495.2 ± 3.9	321
	Histamine	10^{-4}	167.4 ± 14.4	109
	Histamine	10^{-3}	162.1 ± 4.6	105
	A23187	10^{-5}	598.7 ± 4.1	388
4.	None	—	112.1 ± 5.8	100
	Carbachol	10^{-3}	393.9 ± 15.5	351
	Histamine	10^{-3}	132.8 ± 8.7	118
	VIP	10^{-8}	254.0 ± 12.3	227
	VIP	10^{-7}	264.1 ± 3.5	236
	VIP	10^{-6}	281.1 ± 8.8	251
5.	None	–	160.3 ± 10.0	100
	Carbachol	10^{-3}	642.0 ± 28.9	401
	VIP	10^{-7}	444.3 ± 32.2	277
	PGE_1	10^{-6}	543.5 ± 31.7	339
	PGE_1	10^{-5}	729.6 ± 30.4	455

(2) exocytosis of secretory proteins by other cell types was examined. To perform these experiments, potential secretagogues were dissolved directly in the secretion medium, except for A23187, PGE_1 and PMA, which were dissolved initially at a concentration of 10 mM in dimethyl sulphoxide and then diluted in secretion medium for testing. In control incubations, the small amount of dimethyl sulphoxide (0.1% at most) present in the secretion assay mixture did not affect mucin release. Also, after incubation with all compounds, cell viability as determined by Trypan Blue exclusion was always found to be greater than 95%. Carbachol was tested first since it accelerates mucin exocytosis from colonic crypt goblet cells (Specian & Neutra, 1980, 1982) and from mucin-secreting clones derived from the HT29 cell line (Roumagnac & Laboisse, 1987; Phillips *et al.*, 1988), and also causes chloride secretion from T84 cells (Dharmsathaphorn & Pandol, 1986). Carbachol (1 mM) caused the amount of mucin appearing in the medium to increase linearly with time for 30–60 min at a level about 3–4 times that of the unstimulated output (results not shown). Since data from several time-course experiments revealed that the rate of mucin secretion sometimes decreased after 30 min, incubation periods of 30 min were chosen for further experiments. Carbachol stimulated mucin release significantly ($P < 0.001$) over this time period. By including atropine (10 μM) in the incubation medium the stimulation could be abolished without affecting the level of basal secretion. Thus carbachol is acting through a receptor-mediated process. The same pattern of secretory responses was obtained when the

Table 3. Effect of VIP, carbachol and cholera toxin on mucin release

Conditions were the same as described in the legend to Table 2. Results are means ± S.E.M.

	Secretagogue	Mucin protein (pg/μg of cell protein)	Predicted mean value of an additive response (pg/μg)
(*a*)	None	100.5 ± 8.8	–
	Carbachol (1 mM)	494.0 ± 16.0	–
	VIP (10 nM)	213.6 ± 12.9	–
	Carbachol (1 mM) + VIP (10 nM)	597.4 ± 20.9	607.1
	VIP (0.1 μM)	238.2 ± 11.1	—
	Carbachol (1 mM) + VIP (0.1 μM)	681.9 ± 19.5	631.7
	VIP (1 μM)	280.4 ± 12.3	—
	Carbachol (1 mM) + VIP (1 μM)	620.6 ± 24.7	673.9
(*b*)	None	110.6 ± 5.1	–
	Carbachol (1 mM)	544.4 ± 30.4	–
	VIP (10 nM)	203.3 ± 12.9	–
	Cholera toxin (10 μg/ml)	124.0 ± 4.0	–
	Cholera toxin (20 μg/ml)	106.4 ± 5.2	–

antibody to colonic mucin replaced the anti-(SI mucin) antibody in the immunoassay (results not shown). The total mucin content of the cells, including any mucin which might have adhered to the surface, was measured at the start and finish of the incubation period. The amount of mucin secreted in response to carbachol treatment for 30 min was about 2% of the total cellular mucin at the start of the incubation, and no significant change in total mucin content was observed at the end of the incubation. One experiment, in which electron microscopy followed by Immunogold localization of anti-mucin antibody on sections of fixed cells which had been treated previously for 30 min with carbachol, was carried out. Mucous cells showed evidence of granule fusion and release of mucin at the apical surface. Apical cavitation, as described by Specian & Neutra (1980) for crypt goblet cells after treatment with cholinergic agents, was not observed.

Cell cultures were then exposed to several other compounds which are known to cause chloride secretion from T84 monolayers (Dharmsathaphorn *et al.*, 1984; Wasserman *et al.*, 1988). Each agent was tested at several concentrations in at least three separate experiments and the amounts of mucin released after 30 min of incubation were measured. The same pattern of results was obtained in each case, and data from typical experiments are shown in Table 2. Results show that A23187, like carbachol, caused release of mucin from T84 cultures. Raising the A23187 concentration from 1 μM to 10 μM or the carbachol concentration from 0.1 mM to 1 mM increased the level of response. At the concentrations used, both of these compounds are known to increase cytosolic levels of free Ca^{2+} and stimulate chloride secretion by T84 monolayers (Cartwright *et al.*, 1985; Dharmsathaphorn & Pandol, 1986). However, histamine, which has been reported to act like carbachol in elevating free cytosolic Ca^{2+} and stimulating chloride secretion maximally at a concentration of 0.1 mM (Wasserman *et al.*, 1988) had no effect on mucin release even at 1 mM. VIP and PGE_1, which are known to stimulate chloride secretion via a cyclic-AMP-mediated pathway (Dharmsathaphorn *et al.*, 1985; Weymer *et al.*, 1985) also caused mucin release (Table 2), with PGE_1 more effective than VIP at the concentrations used. Increasing the concentration of VIP from 10 nM to 1 μM appeared to have little effect on the amount of mucin appearing in the medium. Also when carbachol (1 mM)

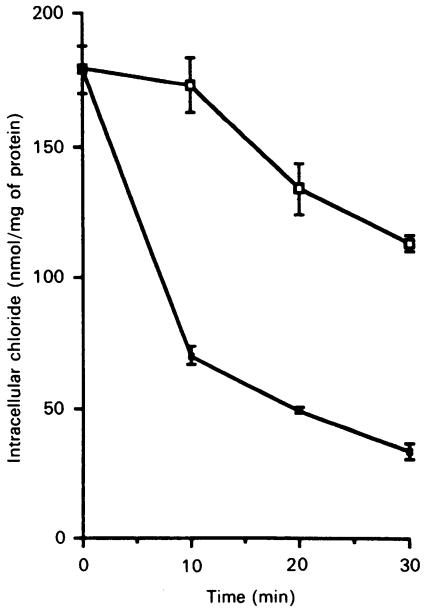

Fig. 5. Acceleration of the rate of $^{36}Cl^-$ efflux from T84 cells by VIP

The loss of intracellular isotope from cells preloaded with $^{36}Cl^-$ was measured at various times in the presence (■) or absence (□) of VIP (0.1 μM). Results are expressed as the mean remaining intracellular $^{36}Cl^-$ (nmol/mg of cell protein) ± S.E.M. of six wells of cells at each time point.

was added together with VIP at various concentrations (10 nM–1 μM), the amount of mucin appearing in the culture medium was approximately equal to the sum of the amounts released in response to each alone (Table 3*a*).

Since the culture conditions used for our experiments are different from those used by Dharmsathaphorn and co-workers for studies of chloride secretion by T84 cells, we performed experiments to show that chloride secretion by cells grown under our culture conditions was responsive to secretagogue stimulation. Using the method of Mandel *et al.* (1986), T84 cells were loaded with $^{36}Cl^-$ and chloride efflux was measured after secretagogue addition. VIP (0.1 μM) accelerated chloride efflux (Fig. 5) at a level similar to that described for T84 monolayers (Mandel *et al.*, 1986). A23187 (10 μM) was about one-third as effective as VIP over the 30 min period (results not shown).

Cholera toxin causes both fluid and mucin secretion from intestinal cells (Forstner *et al.*, 1981; Roomi *et al.*, 1984), and its effect on mucin secretion by T84 cells was examined. Table 3(*b*) shows that the toxin at concentrations of 10 and 20 μg/ml, unlike carbachol and VIP, had no effect on mucin release over a 30 min incubation period.

The calcium ionophore A23187 stimulates secretion from a number of exocrine cell types by moving Ca^{2+} into the cytosol, and the phorbol ester, PMA, can substitute for diacylglycerol in activating protein kinase C (Rink & Knight, 1988) PMA has been reported to stimulate mucin secretion from rat submandibular gland cells and rabbit gastric mucosal explants, and a synergistic relationship between PMA and Ca^{2+} in this process has been suggested (Fleming *et al.*, 1986; Seidler & Sewing, 1989). Therefore we examined the effects of PMA alone and of PMA together with A23187 on mucin secretion by T84 cells. PMA (0.1 and 10 μM) significantly stimulated mucin release ($P < 0.001$) over control levels after a 30 min incubation (Table 4). When PMA and A23187 were added together at various concentrations (Table 4), all combinations stimulated secretion to a level predicted for at least an additive response. A

Table 4. Effects of PMA and A23187 on mucin release

Conditions were the same as described in Table 2. Results are means ± S.E.M.

Expt.	Secretagogue	Mucin protein (pg/μg of cell protein)	Predicted mean value of an additive response (pg/μg)
1	None	101.7±7.7	–
	A23187 (2 μM)	229.3±10.5	–
	PMA (10 nM)	145.2±10.2	–
	PMA (10 nM)+A23187 (2 μM)	329.6±7.0	272.8
	PMA (10 μM)	461.5±13.2	–
	PMA (10 μM)+A23187 (2 μM)	592.3±20.3	589.1
2	None	141.2±4.3	–
	A23187 (1 μM)	245.7±11.9	–
	PMA (0.1 μM)	567.9±39.0	–
	PMA (0.1 μM)+A23187 (1 μM)	884.3±40.5	672.4
3	None	132.4±6.3	–
	A23187 (10 μM)	557.9±25.0	–
	PMA (10 nM)	154.1±11.5	–
	PMA (10 nM)+A23187 (10 μM)	1095.5±36.3	579.6

greater than additive response was obtained with a high concentration (10 μM) of A23187, combined with a concentration (10 nM) of PMA which had relatively little effect on mucin secretion when used alone.

DISCUSSION

Although T84 cells in culture were initially reported to have intracellular mucin-containing granules by immunofluorescence (Brady *et al.*, 1984), a subsequent exhaustive light and electron microscopic study of monolayer cultures (Madara *et al.*, 1987) makes no mention of goblet cell differentiation or mucin granules. Results presented in this study show that the T84 human colonic epithelial cell line, when cultured as a mixture of spheroid aggregates and monolayers, contained goblet-like cells which secreted mucin into the culture medium and were responsive to stimulation. From PAS staining and electron microscopy of fixed culture sections, mucin-producing cells were found mainly among the cell aggregates where early glandular formation was sometimes apparent. Mucin-secreting cells of another human colonic adenocarcinoma cell line, LS174T, are also known to grow in a similar manner in culture (Kuan *et al.*, 1987). When variants were isolated from this cell line, those containing high levels of mucin also tended to form aggregates, whereas one which produced little mucin formed well-spread monolayers in culture. These two cell lines therefore seem to be capable of goblet cell differentiation and do so principally when cell aggregation is occurring.

Mucins from T84 cells and human small intestine compete for binding to an antibody raised in rabbits against the SI mucin. The T84 cell mucin also reacts with an antibody raised against human colonic mucin, showing that it has a class of antigenic determinants which are widely distributed in the intestinal tract. Immunogold labelling with the SI antibody was highly specific, being restricted to typical mucin granules, surface mucus and Golgi areas. When mucin was extracted from the cells and purified, the immunoreactive material was of high molecular mass and had an amino acid profile very similar to that of mucins prepared from human colon, small intestine and another human

colonic adenocarcinoma cell line. No evidence was obtained of anti-(SI mucin) antibody cross-reactivity with proteoglycans, but, as one would anticipate of a mucin, treatment by proteolysis and β-elimination diminished its reactivity with antibody. The sensitive immunoassay we have developed for measuring mucins secreted by cultured cells therefore appears to be highly specific.

From our survey of potential secretagogues, it appears that mucin release from T84 cells is responsive to a number of agents which are known to influence secretion via several different mechanisms. Carbachol, which elevates the cytosolic level of free Ca^{2+}, was stimulatory (Table 2) in agreement with reports of cholinergic action on mucin secretion from colonic crypt goblet cells (Specian & Neutra, 1980, 1982) and HT29 mucin-secreting subclones (Roumagnac & Laboisse, 1987; Phillips *et al.*, 1988). The calcium ionophore A23187, which also raises intracellular Ca^{2+}, caused mucin release (Table 2) and has been reported to stimulate mucin secretion from guinea pig and rabbit mucosal explants (Rutten & Ito, 1985; Seidler & Sewing, 1989). PGE_1, which is known to increase cyclic AMP levels and cause secretion of both intestinal mucus and water (see review by Neutra & Forstner, 1987), was also stimulatory (Table 2). VIP, which also acts via cyclic AMP and is a potent stimulator of intestinal electrolyte and water secretion, caused some mucin release (Table 2) but was less effective than the other three compounds mentioned above. The secretory effect of VIP was unexpected. It has recently been reported that the Cl.16E colonic cell line secretes mucin in response to carbachol (1 mM) but not to VIP even at a concentration of 1 μM, although VIP raised the level of cyclic AMP in these cells (LaBurthe *et al.*, 1989). However, when VIP was added together with carbachol, mucin secretion was potentiated, suggesting an interaction between cyclic AMP and Ca^{2+} systems in mucus secretion. In our experiments using the T84 cell line and similar concentrations of secretagogues, VIP and carbachol produced an additive secretory response (Table 3*a*), indicating that mucin release was probably affected by both cyclic AMP and Ca^{2+}-dependent stimuli. Stimulation of secretion by PGE_1 provides additional evidence for a cyclic-AMP-dependent intermediary pathway. The amount of mucin released into the medium in response to carbachol (1 mM) during the 30 min incubation was small, about 2% of the mucin content of the T84 cultures. At this level of secretion, one might expect little stimulation of mucin synthesis, and indeed no significant change in total mucin content was found at the end of the incubation period. This is in agreement with observations of LaBurthe *et al.* (1989), who showed that addition of carbachol to cell cultures of the Cl.16E colonic cell line stimulated mucin secretion but did not increase glycoprotein synthesis over a 45 min incubation period.

The phorbol ester PMA stimulated mucin release, suggesting that protein kinase C was involved (Table 4). PMA and A23187 stimulated secretion at least additively, indicating that they were capable of mediating secretion by independent mechanisms. When PMA at a concentration (10 nM) which was not an effective stimulant for mucin release was combined with A23187 at a concentration (10 μM) which stimulated mucus release strongly, a potentiated response was obtained. However, a high concentration (10 μM) of PMA and a lower concentration (2 μM) of A23187 produced an additive response. These results suggest that the cytosolic Ca^{2+} concentration is an important modulator of phorbol ester activity and are consistent with reports of an effect of Ca^{2+} on phorbol-ester-stimulated mucus secretion from rabbit mucosal explants (Seidler & Sewing, 1989) and rat submandibular gland cells (Fleming *et al.*, 1986). The A23187 results indicate that an increase in cytosolic Ca^{2+} stimulates mucin release. Free cytosolic Ca^{2+} has also been implicated as a second messenger mediating the stimulatory effects of histamine, carbachol and A23187 on chloride secretion by T84 cells (Dharmsathaphorn & Pandol, 1986; Wasserman *et al.*, 1988). Histamine however, unlike carbachol and A23187, failed to cause mucin release. The reasons for this discrepancy are not clear. Perhaps goblet cell differentiation may be associated with loss of histamine receptors. Absence of cellular receptors might also account for the failure of cholera toxin to stimulate mucin secretion, even though VIP and PGE_1, which also act via a cyclic AMP-dependent process, were effective.

Since our T84 cultures contain chloride-secreting cells, stimulation of chloride and hence water secretion could perhaps result in 'washout' of presecreted mucin from the surface of cells, making it difficult to assess true mucin secretion. Two of our observations suggest, however, that 'washout', if it occurs, plays a relatively insignificant role in our results. Firstly, histamine, which stimulates chloride secretion optimally at 0.1 mM (Wasserman *et al.*, 1988) has no effect on mucin release even at a concentration of 1 mM (Table 2). Chloride-dependent fluid flow therefore does not appear to be sufficient, by itself, to increase mucin output in our system. Secondly, VIP, which is a potent stimulator of chloride secretion and more effective than A23187 (Cartwright *et al.*, 1985), was a less effective secretagogue for mucin than was A23187. Thus the ability to secrete chloride is not well correlated with the ability to secrete mucin. Also we have shown elsewhere (Marcon *et al.*, 1990) that bumetanide, which blocks the T84 sodium/potassium/chloride cotransporter and inhibits the chloride secretory response to VIP and A23187 (Dharmsathaphorn *et al.*, 1985), had no effect on A23187-stimulated mucin secretion from T84 cells. These observations provide evidence for concluding that mucin secretion and chloride secretion are independent activities in the T84 cell line. This cell line therefore possesses two functions typical of intestinal and colonic glandular epithelia. Simultaneously increased secretion of fluid and mucin is often a feature of intestinal inflammatory diseases and infections, suggesting that these two functions may participate in a co-ordinated response to intestinal injury. Since the T84 cell line appears to have retained many of the regulatory mechanisms of colonic tissue, it seems ideally suited to studying mechanisms by which such co-ordination can be achieved.

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REFERENCES

Aminoff, D., Gathmann, W. D., McLean, C. M. & Yadomae, T. (1980) Anal. Biochem. **101**, 44–53

Augeron, C. & Laboisse, C. L. (1984) Cancer Res. **44**, 3961–3969

Brady, R. C., Karnaky, K. J. & Dedman, J. R. (1984) Exp. Cell Res. **150**, 141–151

Byrd, J. C., Nardelli, J., Siddiqui, B. & Kim, Y. S. (1988) Cancer Res. **48**, 6678–6685

Cartwright, C. A., McRoberts, J. A., Mandel, K. G. & Dharmsathaphorn, K. (1985) J. Clin. Invest. **76**, 1837–1842

Dharmsathaphorn, K. & Pandol, S. J. (1986) J. Clin. Invest. **77**, 348–354

Dharmsathaphorn, K., McRoberts, J. A., Mandel, K. G., Tisdale, L. D. & Masui, H., (1984) Am. J. Physiol. **246**, G204–G208

Dharmsathaphorn, K., Mandel, K. G., Masui, H. & McRoberts, J. A. (1985) J. Clin. Invest. **75**, 462–471

Fleming, N., Bilan, P. T. & Sliwinski-Lis, E. (1986) Pflügers Arch. **406**, 6–11

Forstner, G. G. & Forstner, J. F. (1986) in Molecular and Cellular Basis of Digestion (Desnuelle, P., Sjöström, H. & Norén, O., eds.), pp. 125–143, Elsevier, Amsterdam

Forstner, J. F., Roomi, N. W., Fahim, R. E. F. & Forstner, G. G. (1981) Am. J. Physiol. **240**, G10–G16

Hand, P. H., Colcher, D., Salomon, D., Ridge, J., Noguchi, P. & Schlom, J. (1985) Cancer Res. **45**, 833–840

Huet, C., Sahuquillo-Merino, C., Coudrier, E. & Louvard, D. (1987) J. Cell Biol. **105**, 345–357
Kim, K. C., Rearick, J. I., Nettesheim, P. & Jetten, A. M. (1985) J. Biol. Chem. **260**, 4021–4027
Kuan, S. F., Byrd, J. C., Basbaum, C. B. & Kim, Y. S. (1987) Cancer Res. **47**, 5715–5724
LaBurthe, M., Augeron, C., Rouyer-Fessard, C., Roumagnac, I., Maoret, J. J., Grasset, E. & Laboisse, C. (1989) Am. J. Physiol **256**, G443–G450
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Madara, J. L., Stafford, J., Dharmsathaphorn, K. & Carlson, S. (1987) Gastroenterology **92**, 1133–1145
Mandel, K. G., Dharmsathaphorn, K. & McRoberts, J. A. (1986) J. Biol. Chem. **261**, 704–712
Mantle, M., Forstner, G. G. & Forstner, J. F. (1984*a*) Biochem. J., **224**, 345–354
Mantle, M., Forstner, G. G. & Forstner, J. F. (1984*b*) Biochem. J. **217**, 159–167
Marcon, M. A., McCool, D. J., Forstner, J. F. & Forstner, G. G. (1990) Biochim. Biophys. Acta, in the press
Murakami, H. & Masui, H. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 3464–3468
Neutra, M. R. & Forstner, J. F. (1987) in Physiology of the Gastrointestinal Tract (Johnson L. R., ed.), vol. 2, pp. 975–1009, Raven Press, New York
Pearse, A. G. (1968) Histochemistry, Theoretical and Applied, 3rd ed., vol. 1, pp. 659–673. J. and A. Churchill, London
Phillips, T. E., Huet, C., Bilbo, P. R., Podolsky, D. K., Louvard, D. & Neutra, M. R. (1988) Gastroenterology **94**, 1390–1403
Qureshi, R., Forstner, G. G. & Forstner, J. F. (1979) J. Clin. Invest. **64**, 1149–1156
Rink, T. J. & Knight, D. E. (1988) J. Exp. Biol. **139**, 1–30
Roomi, N., LaBurthe, M., Fleming, N., Crowther, R. & Forstner, J. (1984) Am. J. Physiol. **247**, G140–G148
Roumagnac, I. & Laboisse, C. (1987) Biol. Cell **61**, 65–68
Rutten, M. J. & Ito, S. (1985) Fed. Proc. Fed. Am. Soc. Exp. Biol. **44**, 618 (Abstr. 1212)
Seidler, U. & Sewing, K.-F. R. (1989) Am. J. Physiol. **256**, G739–G746
Specian, R. D. & Neutra, M. R. (1980) J. Cell Biol. **85**, 626–640
Specian, R. D. & Neutra, M. R. (1982) Am. J. Physiol. **242**, G370–G379
Wasserman, S. I., Barrett, K. E., Huott, P. A., Beuerlein, G., Kagnoff, M. F. & Dharmsathaphorn, K. (1988) Am. J. Physiol. **254**, C53–C62
Wesley, A., Mantle, M., Man, D., Qureshi, R., Forstner, G. & Forstner, J. (1985) J. Biol. Chem. **260**, 7955–7959
Weymer, A., Huott, P., Liu, W., McRoberts, J. A. & Dharmsathaphorn, K. (1985) J. Clin. Invest. **76**, 1828–1836

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